DESCRIPTION

POLYPEPTIDE HAVING AN ACTIVITY TO SUPPORT PROLIFERATION OR SURVIVAL OF HEMATOPOIETIC STEM CELL OR HEMATOPOIETIC PROGENITOR CELL, AND DNA CODING FOR THE SAME

Technical Field

The present invention relates to a polypeptide having an activity to support proliferation or survival of hematopoietic stem cells or hematopoietic progenitor cells, a DNA coding the polypeptide, and a pharmaceutical composition comprising the polypeptide as active ingredient.

Background Art

Fully differentiated mature hematopoietic cells have limited short lives. Homeostasis of the blood is maintained due to supply of the mature blood cells caused by continuous differentiation of hematopoietic progenitor cells. The hematopoietic progenitor cells are giving rise from more undifferentiated hematopoietic stem cells. The hematopoietic stem cells have potential of differentiating into all of the differentiation lineages (totipotency) and have potential of self-renew with retaining the totipotency so as to supply the hematopoietic cells through life. That is, the hematopoietic stem cells are known to generate totipotent stem cells by the self-renew and to

differentiate in parts to a variety of the mature blood cells through the hematopoietic progenitor cells.

This differentiation of the blood cells is regulated by a variety of cytokines. Erythropoietin is known to promote the differentiation of the erythrocytic lineages. G-CSF and thrombopoietin are also known to promote the differentiation of the neutrophils, and the megakaryocytes and the platelet productive cells, respectively. However, a factor required for the self-renew of the hematopoietic stem cell with retaining the totipotency has not been clear. Although SCF/MGF (Williams, D.E., *Cell*, *63*: 167-174, 1990; Zsebo, K.M., *Cell*, *63*: 213-224, 1990), SCGF (WO98/08869), and the like are reported as growth factors for the hematopoietic stem cells, none of them have potency to sufficiently retain the totipotency of the hematopoietic stem cells. Although attempts to culture the hematopoietic stem cells in the presence of combinations of known cytokines, a system for efficient amplification of the hematopoietic stem cells was not realized (Miller, C. L., *Proc. Natl. Acad. Sci. USA*, *94*: 13648-13653, 1997; Yagi, M., *Proc. Natl. Acad. Sci. USA*, *96*: 8126-8131, 1999; Shih, C.C., *Blood*, *94*: 5 1623-1636, 1999).

On the other hand, attempts to allow the hematopoietic stem cells to survive or proliferate without differentiation by using stromal cells which supply an environment suitable for survival or

proliferation of the hematopoietic stem cells were reported (Moore K.A., *Blood*, *89*: 12, 4337-4347, 1997). In addition, WO99/03980 discloses a stromal cell line capable of supporting proliferation or survival of hematopoietic stem cells and hematopoietic progenitor cells, which are established from an AGM (Aorta-Gonad-Mesonephros) region of a fetal mouse.

It is postulated that there should be more peptides that efficiently facilitate hematopoietic stem cell and progenitor cell amplification by themselves or in combination with stromal cells or stimulating factors such as cytokines, in addition to known factors affecting hematopoietic cells.

Disclosure of Invention

Since the proliferation or survival of hematopoietic stem cells or hematopoietic progenitor cells *in vitro* can be supported by co-culture of stromal cells and hematopoietic stem cells and hematopoietic progenitor cells, the stromal cells are expected to produce factors supporting the proliferation or survival of hematopoietic stem cells or hematopoietic progenitor cells. An object of the present invention is to provide a factor supporting the proliferation or survival of hematopoietic stem cells or hematopoietic progenitor cells, which is derived from the stromal cells.

The inventor of the present invention has assumed

that the mouse stromal cells produce factors supporting the proliferation or survival of hematopoietic stem cells or hematopoietic progenitor cells, as mentioned above. Attention is given that there are two kinds of stromal cells. One has a ability to support the proliferation or survival of hematopoietic stem cells or hematopoietic progenitor cells (hereafter sometimes referred to as "activity to support hematopoietic stem cells"). The other does not have the activity to support hematopoietic stem cells. The inventor of the present invention has assumed that this difference in the ability is due to the fact that expression of genes encoding the factors is increased in the supporting stromal cells and that the expression is low in non-supporting stromal cells. Thus the inventor think it can be found the factors supporting the proliferation or survival of hematopoietic stem cells or hematopoietic progenitor cells among the genes expressed higher in the supporting cells compared to in the non-supporting cells. In this context, the inventor has identified genes of which expressions are high in AGM-s3-A9 cell line which has the activity to support hematopoietic stem cells, and low or undetected in AGM-s3-A7 cell line which does not have the activity to support hematopoietic stem cells, and has determined the activities to support hematopoietic stem cells, of cells in which these gene groups are highly expressed. As a result, the present

invention has been completed.

That is, the present invention provides the followings.

(1) A DNA coding for a polypeptide of the following (A) or (B):

(A) a polypeptide which comprises the amino acid sequence of SEQ ID NO: 48; or

(B) a polypeptide which comprises an amino acid sequence including deletion, substitution or insertion of one or several amino acids in the amino acid sequence as defined in (A), and which has an activity to support proliferation or survival of hematopoietic stem cells or hematopoietic progenitor cells.

(2) The DNA according to (1), which is a DNA of the following (a) or (b):

(a) a DNA which comprises the nucleotide sequence of nucleotides 18 to 746 of SEQ ID NO: 47; or

(b) a DNA which is hybridizable with a DNA comprising the nucleotide sequence as defined in (a) or a prove prepared from said DNA, under the stringent condition, and which has an activity to support proliferation or survival of hematopoietic stem cells or hematopoietic progenitor cells.

(3) The DNA according to (2), the stringent condition is 6 x SSC、5 x Denhardt, 0.5% SDS and 68°C (SSC: 3 M NaCl, 0.3 M sodium citrate; 50 x Denhardt: 1% BSA、1% polyvinyl pyrrolidone, 1% Ficoll 400), or 6 x

SSC, 5 x Denhardt, 0.5% SDS, 50% formamide and 42°C.

(4) A expression vector which comprises the DNA of any one of (1) to (3) in such a manner that the DNA can be expressed.

(5) A cell into which the DNA of any one of (1) to (3) is introduced in such a manner that the DNA can be expressed.

(6) A polypeptide which is an expression product of the DNA of any one of (1) to (3), the polypeptide having an activity to support proliferation or survival of hematopoietic stem cells or hematopoietic progenitor cells.

(7) The polypeptide according to (6), which comprises the amino acid sequence of SEQ ID NO: 48, or an amino acid sequence including deletion, substitution or insertion of one or several amino acids in the amino acid sequence.

(8) The polypeptide according to (6) or (7), which is modified with one or more modifying agents selected from the group consisting of polyethylene glycol (PEG), dextran, poly(N-vinyl-pyrrolidone), polypropylene glycol homopoymer, copolymer of polypropylene oxide/ethylene oxide, polyoxyethylated polyol and polyvinyl alcohol.

(9) An monoclonal antibody which binds to the polypeptide of any one of (6) to (8).

(10) A method for supporting proliferation or

survival of hematopoietic stem cells or hematopoietic progenitor cells, comprising the step of co-culturing stromal cells in which a DNA coding for a polypeptide of the following (A) or (B) is expressed, with hematopoietic stem cells or progenitor cells,

(A) a polypeptide which comprises the amino acid sequence of SEQ ID NO: 48; or

(B) a polypeptide which comprises an amino acid sequence including deletion, substitution or insertion of one or several amino acids in the amino acid sequence as defined in (A), and which has an activity to support proliferation or survival of hematopoietic stem cells or hematopoietic progenitor cells.

(11) The method according to (10), wherein the DNA is a DNA of the following (a) or (b):

(a) a DNA which comprises the nucleotide sequence of nucleotides 18 to 746 of SEQ ID NO: 47; or

(b) a DNA which is hybridizable with a DNA comprising the nucleotide sequence as defined in (a) or a prove prepared from said DNA, under the stringent condition, and which has an activity to support proliferation or survival of hematopoietic stem cells or hematopoietic progenitor cells.

(12) A method for supporting proliferation or survival of hematopoietic stem cells or hematopoietic progenitor cells, comprising the step of culturing hematopoietic stem cells or progenitor cells in the

presence of a polypeptide of the following (A) or (B), said polypeptide having an activity to support proliferation or survival of hematopoietic stem cells or hematopoietic progenitor cells when the hematopoietic stem cells or hematopoietic progenitor cells are cultured in the presence of the polypeptide,

(A) a polypeptide which comprises the amino acid sequence of SEQ ID NO: 48; or

(B) a polypeptide which comprises an amino acid sequence including deletion, substitution or insertion of one or several amino acids in the amino acid sequence as defined in (A), and which has an activity to support proliferation or survival of hematopoietic stem cells or hematopoietic progenitor cells.

(13) A pharmaceutical composition having an effect to support proliferation or survival of hematopoietic stem cells or hematopoietic progenitor cells, which comprises an effective amount of a polypeptide of the following (A) or (B), said polypeptide having an activity to support proliferation or survival of hematopoietic stem cells or hematopoietic progenitor cells when hematopoietic stem cells or hematopoietic progenitor cells are cultured in the presence of the polypeptide,

(A) a polypeptide which comprises the amino acid sequence of SEQ ID NO: 48; or

(B) a polypeptide which comprises an amino acid

sequence including deletion, substitution or insertion of one or several amino acids in the amino acid sequence as defined in (A), and which has an activity to support proliferation or survival of hematopoietic stem cells or hematopoietic progenitor cells.

Terms used in this specification are defined as follows.

A hematopoietic stem cell is defined as a cell having totipotency, that is, ability to differentiate into all the cell lineages of the blood cells, and having a potency of self-renew with retaining the totipotency. A hematopoietic progenitor cell is defined as a cell which can differentiate a single cell lineage of the blood cell or plural cell lineages but cannot differentiate into all of the cell lineages. A stromal cell is defined as a cell which can be co-cultured together with the hematopoietic stem cells to construct a hematopoietic environment simulating *in vivo* hematopoietic environment *in vitro*. Cells derived from any origin can be used as long as the cells can be co-cultured with the hematopoietic cells *in vitro*.

Erythrocyte progenitor cells hardly survive and proliferate in *in vitro* culture environments and rapidly disappear. If the survival and proliferation of the erythrocyte progenitor cells are observed, continuous production of the erythrocyte progenitor cells is predicted to occur due to the survival and proliferation

of the more immature hematopoietic stem cells or the hematopoietic progenitor cells. Therefore, in an assessment system of human hematopoietic stem cells, proliferation of hematopoietic stem cells or immature hematopoietic progenitor cells can be determined by using the survival and proliferation of the erythrocyte progenitor cells (BFU-E, CFU-E, and CFU-E mix) as an index.

Brief Description of Drawings

Fig. 1 shows proliferation statuses of hematopoietic stem cells and hematopoietic progenitor cells determined by a clonogenic assay after co-culture of CD34-positive hematopoietic stem cells with AGM-s3 subclone A9, A7, or D11 cells for two weeks.

Fig. 2 shows proliferation statuses of hematopoietic stem cells and hematopoietic progenitor cells determined by a clonogenic assay after co-culture of CD34-positive hematopoietic stem cells with AGM-s3 subclone A9, A7, or OP9 cells for two weeks.

Fig. 3 shows time course of donor derived lymphoid lineage cells or myeloid lineage cells reconstitution in irradiated recipient mice that received the hematopoietic stem cells co-cultured with stromal cells.

Fig. 4 shows proliferation statuses of hematopoietic stem cells and hematopoietic progenitor cells determined by a clonogenic assay after co-culture of CD34-positive

hematopoietic stem cells with AGM-S3-A9 cells in which a gene SCR-2 is highly expressed (A9/SCR-2), AGM-S3-A9 cells into which a control vector is introduced (A9/pMXIG) or AGM-S3-A9 cells (A9) for two weeks.

Fig. 5 shows proliferation statuses of hematopoietic stem cells and hematopoietic progenitor cells determined by a clonogenic assay after co-culture of CD34-positive hematopoietic stem cells with AGM-S3-A7 cells in which a gene SCR-2 is highly expressed (A7/SCR-2), AGM-S3-A7 cells into which a control vector is introduced (A7/pMXIG) or AGM-S3-A7 cells (A7) for two weeks.

Fig. 6 shows time course of donor derived lymphoid lineage cells or myeloid lineage cells reconstitution in peripheral blood of irradiated recipient mice that received the hematopoietic stem cells co-cultured with AGM-S3-A7 cells in which a gene SCR-3 is highly expressed (A7/SCR-3), AGM-S3-A7 cells into which a control vector is introduced (A7/pMXIG) or AGM-S3-A7 cells.

Fig. 7 shows proliferation statuses of hematopoietic stem cells and hematopoietic progenitor cells determined by a clonogenic assay after co-culture of CD34-positive hematopoietic stem cells with AGM-S3-A9 cells in which a gene SCR-4 is highly expressed (A9/SCR-4), AGM-S3-A9 cells into which a control vector is introduced (A9/pMXIG) or AGM-S3-A9 cells (A9) for two weeks.

Fig. 8 shows time course of donor derived lymphoid

lineage cells or myeloid lineage cells reconstitution in peripheral blood of irradiated recipient mice that received the hematopoietic stem cells co-cultured with AGM-S3-A7 cells in which a gene SCR-5 is highly expressed (A7/SCR-5), AGM-S3-A7 cells into which a control vector is introduced (A7/pMXIG) or AGM-S3-A7 cells.

Fig. 9 shows proliferation statuses of hematopoietic stem cells and hematopoietic progenitor cells determined by a clonogenic assay after co-culture of CD34-positive hematopoietic stem cells with AGM-S3-A9 cells in which a gene SCR-6 is highly expressed (A9/SCR-6), AGM-S3-A9 cells into which a control vector is introduced (A9/pMXIG) or AGM-S3-A9 cells (A9) for two weeks.

Fig. 10 shows proliferation statuses of hematopoietic stem cells and hematopoietic progenitor cells determined by a clonogenic assay after co-culture of CD34-positive hematopoietic stem cells with AGM-S3-A9 cells in which a gene SCR-7 is highly expressed (A9/SCR-7), AGM-S3-A9 cells into which a control vector is introduced (A9/pMXIG) or AGM-S3-A9 cells (A9) for two weeks.

Fig. 11 shows proliferation statuses of hematopoietic stem cells and hematopoietic progenitor cells determined by a clonogenic assay after co-culture of CD34-positive hematopoietic stem cells with AGM-S3-A9 cells in which a gene SCR-8 is highly expressed (A9/SCR-

8), AGM-S3-A9 cells into which a control vector is introduced (A9/pMXIG) or AGM-S3-A9 cells (A9) for two weeks.

Best Mode for Carrying Out the Invention

Hereafter, the present invention will be described in detail below.

The following genes are those identified as genes of which expressions are high in AGM-s3-A9 cell line which has the activity to support hematopoietic stem cells, and low or undetected in AGM-s3-A7 cell line which does not have the activity to support hematopoietic stem cells, and determined to have the activities to support hematopoietic stem cells, of cells in which these gene groups are highly expressed.

Gene SCR-2

The gene is the same gene as a mouse gene, *Mus musculus* glypican-1 (GPC-1) of a GenBank accession number AF185613.

The nuclotide sequence of the gene from mouse and the amino acid sequence deduced from the nucleotide sequence are shown in SEQ ID NO: 8. Only the amino acid sequence is shown in SEQ ID NO: 9.

The human amino acid sequence of GPC-1 is recorded in GenBank under an accession number P35052, and the human nucleotide sequence of GPC-1 is recorded in

GenBank database under an accession number AX020122. It is predicted that the similar activity is detected in the human gene.

The nucleotide sequence of the gene from human and the amino acid sequence deduced from the nucleotide sequence are shown in SEQ ID NO: 10. Only the amino acid sequence is shown in SEQ ID NO: 11.

Glypican is a major hepran sulfate proteoglycan existing on a cell surface, and have a characteristic structure such as cysteine rich globular domain, short glycosaminoglycan binding domain, glycosylphosphatidyl-inositol membrane binding domain. Six family genes from glypican-1 to glypican-6 have been found (J Biol Chem 1999 Sep 17;274(38):26968-77、Glypican-6, a new member of the glypican family of cell surface heparan sulfate proteoglycans. Veugelers M, De Cat B, Ceulemans H, Bruystens AM, Coomans C, Durr J, Vermeesch J, Marynen P, David G).

With respect to biological activities of GPC-1, there are a number of reports: To regulate growth stimulating activity of heparin binding growth factors (fibroblast growth factor 2 (FGF2), heparin-binding EGF-like growth factor (HB-EGF)) to promote proliferation of cancer cells showing autocrine proliferation by stimulation by the growth factors (J Clin Invest 1998 Nov 1; 102(9):1662-73, The cell-surface heparan sulfate proteoglycan glypican-1 regulates growth factor action

in pancreatic carcinoma cells and is overexpressed in human pancreatic cancer., Kleeff J, Ishiwata T, Kumbasar A, Friess H, Buchler MW, Lander AD, Korc M).

To bind HGF (hepatocyte groth factor) to promote reactivity with cytokines, of antigen-specific B cells. To participate in association of a cell with an adhesive molecule to involve in invasion of the cell (J Biol Chem 1998 Aug 28;273(35):22825-32, Heparan sulfate proteoglycans as adhesive and anti-invasive molecules. Syndecans and glypican have distinct functions., Liu W, Litwack ED, Stanley MJ, Langford JK, Lander AD, Sanderson RD). These findings show that GPC-1 involves in activity expression of various cell-stimulating factors. Also, there is a report that expression of the glypican family gene in bone marrow is confirmed (Biochem J 1999 Nov 1;343 Pt 3:663-8, Expression of proteoglycan core proteins in human bone marrow stroma., Schofield KP, Gallagher JT, David G). However, in these reports, it is not described about effects of GPC-1 on hematopoietic stem cells or hematopoietic progenitor cells.

Gene SCR-3

The gene is the same gene as mouse genes, *Mus musculus* chemokine MMRP2 mRNA of a GenBank accession number U15209, *Mus musculus* C10-like chemokine mRNA of U19482 and mouse macrophage inflammatory protein-1gamma

mRNA of U49513.

The nuclotide sequence of the gene from mouse and the amino acid sequence deduced from the nucleotide sequence are shown in SEQ ID NO: 12. Only the amino acid sequence is shown in SEQ ID NO: 13.

Gene SCR-4

The nuclotide sequence of the gene from mouse and the amino acid sequence deduced from the nucleotide sequence are shown in SEQ ID NO: 14. Only the amino acid sequence is shown in SEQ ID NO: 15.

It has been found that the sequence has a high homology to *Homo sapiens* clone 25077 mRNA of a GenBank accession number AF131820, and that it is considered to be a mouse ortholog. This sequence is described in WO 00/66784.

The nuclotide sequence of the gene from human and the amino acid sequence deduced from the nucleotide sequence are shown in SEQ ID NO: 16. Only the amino acid sequence is shown in SEQ ID NO: 17.

Gene SCR-5

The nuclotide sequence of the gene from mouse and the amino acid sequence deduced from the nucleotide sequence are shown in SEQ ID NO: 18. Only the amino acid sequence is shown in SEQ ID NO: 19.

It has been found that the sequence has a high

homology with *Homo sapiens* esophageal cancer related gene 4 portein (ECRG4) mRNA of a GenBank accession number AF325503, and that it is considered to be a mouse ortholog of AF325503.

The nuclotide sequence of the gene from human and the amino acid sequence deduced from the nucleotide sequence are shown in SEQ ID NO: 20. Only the amino acid sequence is shown in SEQ ID NO: 21.

Gene SCR-6

The nuclotide sequence of the gene from mouse and the amino acid sequence deduced from the nucleotide sequence are shown in SEQ ID NO: 22. Only the amino acid sequence is shown in SEQ ID NO: 23.

The nuclotide sequence of the gene from human and the amino acid sequence deduced from the nucleotide sequence are shown in SEQ ID NO: 47. Only the amino acid sequence is shown in SEQ ID NO: 48.

Gene SCR-7

The nuclotide sequence of the gene from mouse and the amino acid sequence deduced from the nucleotide sequence are shown in SEQ ID NO: 24. Only the amino acid sequence is shown in SEQ ID NO: 25.

Gene SCR-8

The gene is the same gene as *Mus musculus* mRNA for

ADAM23 of a GenBank accession number AB009673.

The nuclotide sequence of the gene from mouse and the amino acid sequence deduced from the nucleotide sequence are shown in SEQ ID NO: 26. Only the amino acid sequence is shown in SEQ ID NO: 27.

The sequence has a high homology with a sequence described by JP 11155574-A and the sequence described by JP 11155574-A is considered to be a human ortholog.

The nuclotide sequence of the gene from human and the amino acid sequence deduced from the nucleotide sequence are shown in SEQ ID NO: 28. Only the amino acid sequence is shown in SEQ ID NO: 29.

Polypeptides which are products of these genes have an activity to support proliferation or survival of hematopoietic stem cells or hematopoietic progenitor cells. The expression that a polypeptide has an activity to support proliferation or survival of hematopoietic stem cells or hematopoietic progenitor cells means that proliferation or survival of hematopoietic stem cells or hematopoietic progenitor cells is supported in the presence of the polypeptide or in the presence of stroma cells expressing the polypeptide.

Therefore, the present invention provides use of the polypeptides and DNAs encoding the polypeptides and novel polypeptides among the polypeptides and DNAs

encoding the novel polypeptides.

A stem cell proliferation-supporting factor which is a polypeptide encoded by the DNA can be produced by introducing the DNA into a suitable host to prepare a transformant cell, and allowing the DNA to be expressed in the transformant cell.

The DNA may encode the above described factors which have amino acid sequences including substitution, deletion or insertion of one or several amino acids, as long as the activity of the stem cell proliferation-supporting factor to be encoded is not lost. DNAs encoding substantially equivalent polypeptides to this stem cell proliferation-supporting factor can be obtained by modifying the nucleotide sequences so as to include substitution, deletion, insertion, addition, or inversion of amino acid residues in a specific region using site-directed mutagenesis.

The DNAs including the above described mutation can be expressed in appropriate cells and the activity to support hematopoietic stem cells, of the expressed products can be examined, so that the DNAs encoding the polypeptide having functions which are substantially equivalent to this stem cell proliferation-supporting factor are obtained. In addition, the DNAs encoding substantially equivalently active protein as this stem cell proliferation-supporting factor can be obtained by isolating DNAs which hybridize with DNAs including, for

example, the nucleotide sequence (ORF portion) as described in SEQ ID NO: 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, or 47 from the cells having the DNA, or probes prepared from these DNAs under the stringent condition; and which encode proteins possessing the activity to support hematopoietic stem cells. The length of the probe is usually 30 to 1000 nucleotides. The stringent condition is, for example, one in which DNAs having homology (determinable with homology search in the compare function of DNASIS version 3.7 (Hitachi Software Engineering)) at not less than 70%, preferably at not less than 80%, are hybridized each other and DNAs having less homology than those are not hybridized each other. The above described stringent condition may be 6 × SSC, 5 × Denhardt, 0.5% SDS, 68°C (SSC; 3 M NaCl, 0.3 M sodium citrate) (50 × Denhardt; 1% BSA, 1% polyvinyl pyrrolidone, 1% Ficoll 400) or 6 × SSC, 5 × Deanhardt, 0.5% SDS, 50% Formamide, 42°C, or the like.

Microorganisms such as *Escherichia coli* and yeast, culture cells derived from animals or plants, and the like are used for host cells for expressing the DNA. Preferably, culture cells derived from mammals are used as the host cells. In the case that prokaryotic cells are used as the host cells, the expression is preferably performed in a condition in which a signal peptide region is replaced with a leader sequence suitable for the prokaryotic cells such as β-lactamase (*bla*),

alkaline phosphatase (*phoA*), and outer membrane protein A (*ompA*) and the like, or in a form in which a methionine residue is added to the N-terminal site of the mature protein.

The introduction of the DNA to the host cell can be carried out by, for example, incorporating the DNA into a vector suitable for the host in an expressible form, and introducing the resultant recombinant vector to the host cell.

Examples of the culture cells derived from mammals include CHO cell, 293 cell, COS7 cell, and the like. Gene expression regulatory sequence such as a promoter to express the DNA may be originated from the gene itself, or may be derived from other genes such as cytomegalovirus promoter and elongation factor 1 promoter and the like.

Examples of a vector for animal culture cells include plasmid vectors, retrovirus vectors, adenovirus vectors (Neering, S.J., *Blood*, *88*: 1147, 1996), herpes virus vectors (Dilloo, D., *Blood*, *89*: 119, 1997), HIV vectors, and the like.

In order to introduce the recombinant vector into culture cells, the conventional methods which are usually employed for transformation of culture cells such as calcium phosphate transfection, the liposome method, the DEAE dextran method, the electroporation method and the microinjection method are employed.

The polypeptides of the present invention also comprise polypeptides having amino acid sequences in which one or several amino acids are substituted, deleted or inserted in the amino acid sequence represented in SEQ ID NO: 9, 11, 13, 15, 17, 19, 21, 23, 25, 27 or 29, and having activity to support hematopoietic stem cells in addition to the polypeptides having the amino acid sequence represented in SEQ ID NO: 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, or 48. That is, even if mouse and human stem cell proliferation-supporting factors are modified by substitution, deletion, insertion or the like, polypeptides holding essential functions as a stem cell proliferation-supporting factor can be considered to be substantially equivalent to the stem cell proliferation-supporting factor.

These modified stem cell proliferation-supporting factors can be obtained by treating DNA encoding the stem cell proliferation-supporting factor or host cells including the above mentioned DNA with a mutagen, or by mutating the above mentioned DNA so as to substitute, delete, or insert an amino acid at a specific site using site-directed mutagenesis. The residual of the activity to support the hematopoietic stem cells in the obtained mutant polypeptide is confirmed by tranferring hematopoietic stem cells cultured in the presence of the mutant polypeptides into irradiated mice, and monitering

peripheral hematological cellularity over time, as in the examples described below.

As for the amino acid deletion, the polypeptide may be a fragment which lacks an amino acid sequence at the N-terminal end and/or the C-terminal end. The fragment lacking the amino acid sequence at the N-terminal end and/or the C-terminal end can be obtained by a usual method, and the hematopoietic stem cell-supporting activity of the fragment can be determined by a similar way to that described with respect to the mutated polypeptide. In particular, if there is a portion predicted as a signal sequence or a transmembrane region in the amino acid sequence, a fragment having the hematopoietic stem cell-supporting activity is predicted by using it as an index. For example, a protein encoded by human SCR-8 is a transmembrane protein of type I passing through the membrane once, and it is therefore predicted that even if it made to be a soluble protein lacking the transmembrane region, it has the activity to support to proliferation or survival of hematopoietic stem cells or hematopoietic progenitor cells. The transmembrane region can be predicted with a known program based on the amino acid sequence. For example, if it is predicted with a program called PSORT II (available through the Internet, URL: http://psort.nibb.ac.jp/index.html), the transmembrane region is amino acids at positions 790 to 806 in SEQ ID

NO: 29, and it is predicted that even if a fragment up to position 789, the fragment has activity to support proliferation or survival of hematopoietic stem cells or hematopoietic progenitor cells.

Since the nucleotide sequences of the above described DNAs have been clarified by the present invention, the DNAs can be also obtained by isolating the corresponding DNAs from mouse or human cDNA or chromosome DNA libraries using PCR in which the oligonucleotides prepared based on these nucleotide sequences are used as primers or using hybridization in which the oligonucleotides prepared based on these nucleotide sequences are used as probes.

In order to complete the present invention, the DNAs of the present invention have been isolated from cDNA library of AGM-s3-A9 cells which are a mouse stromal cell line having the activity to support the hematopoietic stem cells, using SBH (Sequencing By Hybridization) method (Drmanac, S., *Nat. Biotechnol., 16.* 54, 1998; Drmanac, R., *Methods. Enzymol., 303,* 165, 1999) as described below. The mouse stromal cell lines having the activity to support the hematopoietic stem cells can be obtained using the method disclosed in WO99/03980 or from Cell Bank of Institute of Physical and Chemical Research (RIKEN) or ATCC.

An outline of SBH method will be described below. Probes having eight or nine nucleotides whose sequences

are different from each other are prepared. When the nucleotide sequences corresponding to those of the probe exist in a targeted gene, the probes can hybridize with the gene. The hybridization can be easily detected with utilization of radio isotope- or fluorescence-labelled probes. Each clone in the library is picked up, and blotted on a membrane. Then, the repeated hybridizations are performed with the each of above described probes, so that one can identify the combination of probes that hybridize to each clone. Since the combination of hybridized probes depends on genes, the combination of probes which hybridize to an identical gene is the same. That is, the same gene can be identified as one group (cluster) according to the the combination of the hybridized probes. The number of clones of each gene in the cDNA library can be determined by classifying each clone in the library based on patterns of the hybridized probes and counting the classified clones. Thus, frequency of expression of each gene in the library can be determined.

cDNA libraries are prepared from cells having an activity to support the hematopoietic stem cells and from cells not having the activity. Clustering is performed for the cDNA libraries. Statuses of expressed genes among cells are compared, so that the genes highly expressed with specificity to the supporting cells are selected. The expression statuses of these genes in

each of above described cells are further examined by Northern blot analysis, so that genes which are highly expressed in the cells having the activity to support the hematopoietic stem cells are obtained.

The above mentioned genes are the genes which are highly expressed with specificity to the supporting cells and which are obtained through the above described process. Full-length genes have been cloned from the cDNA library derived from AGM-s3-A9 cell.

Further, in order to determine an ability of gene products to support hematopoiesis, a gene fragment including gene ORF was transferred into stromal cells using a retrovirus vector, and the change in the activity to support the hematopoietic stem cells of the stromal cells was determined. Specifically, after the stromal cells into which the gene was not introduced or into which a control vector was introduced and those into which the gene was introduced were each co-cultured with the mouse hematopoietic stem cells, the hematopoietic cells were transplanted into irradiated mice. The engraftment of the co-cultured hematopoietic cells in recipient mice were examined. As a result, the mice into which the hematopoietic stem cells co-cultured with the gene-introduced cells were transplanted, showed increased chimerism after the transplantation compared with co-culture with the cells into which the gene was not introduced. This result shows that in the gene-

expressed stromal cells, an activity to support the proliferation or survival of the hematopoietic stem cells or the hematopoietic progenitor cells is increased or imparted. As a result, it has become evident that expression of the above described genes has a function to increase the above described activity in the stromal cells or impart the activity to the stromal cells. Therefore, it is revealed that products of the genes affect hematopoietic stem cells or hematopoietic progenitor cells to show an activity to support the survival or the proliferation thereof, or affect stromal cells to show an activity to increase an activity to support the hematopoietic stem cells therein or impart the activity thereto.

The polypeptides of the present invention can be used as a medicine to proliferate or support human hematopoietic stem cells or human hematopoietic progenitor cells when they affect hematopoietic stem cells or hematopoietic progenitor cells to show an activity to support survival or proliferation thereof, in other words, the polypeptides have an activity to support survival or proliferation of hematopoietic stem cells or hematopoietic progenitor cells if the hematopoietic stem cells or the hematopoietic progenitor cells are cultured in the presence of the polypeptides. The pharmaceutical composition can be used for supporting proliferation or survival of human

hematopoietic stem cells or human hematopoietic progenitor cells *in vitro*. For hematopoietic stem cell transplantation therapies such as peripheral blood stem cell transplantation and cord blood stem cell transplantation, a sufficient amount of the hematopoietic stem cells sometimes cannot be collected and the transplantation may not be performed. Even if the enough amount of the stem cells can not be collected, the enough amount of the hematopoietic stem cells can be obtained and transplanted by amplification of the hematopoietic stem cells *in vitro* using this polypeptides. That is, the hematopoietic stem cells can be amplified without differentiation by culturing the hematopoietic stem cells in culture medium including these polypeptides. It may be considered the hematopoietic stem cells are able to be amplified more efficiently with addition of a variety of cytokines to the medium.

When the hematopoietic stem cells or the hematopoietic progenitor cells are cultured in the medium including the polypeptides of the present invention, the hematopoietic stem cells or the hematopoietic progenitor cells used may be isolated one of these cell types alone or may be both of the cell types. In addition, the cells may include at least the hematopoietic stem cells or the hematopoietic progenitor cells, and include other hematopoietic cells. Further,

it can be used a fraction containing hematopoietic stem cells or progenitor cells fractionated from the cell population that contain the hematopoietic stem cells or progenitor cells.

Examples of sources of the hematopoietic stem cells and the hematopoietic progenitor cells in the method of the present invention include a fetal liver, bone marrow, fetal bone marrow, peripheral blood, the peripheral blood from persons whose stem cells are mobilized by administration of cytokines and/or antitumor drugs, cord blood, and the like of mammals such as human and mouse and the like. Any sources may be used as long as the tissue includes the hematopoietic stem cells.

A culture method using petri dishes and flasks for culture may be employed to culture the hematopoietic stem cells or the hematopoietic progenitor cells. The cultivation of the hematopoietic stem cells and/or progenitor cells may be improved by mechanically controlling medium composition, pH, and the like, and using a bioreactor capable of high density cultivation (Schwartz, *Proc. Natl. Acad. Sci. U.S.A.*, *88*: 6760, 1991; Koller, M.R., *Bio/Technology*, *11*: 358, 1993; Koller, M.R., *Blood*, *82*: 378, 1993; Palsson, B.O., *Bio/Technology*, *11*: 368, 1993).

The stromal cells in which DNAs encoding the polypeptide of the present invention can be obtained as described with respect to the expression of the DNAs.

The co-culture of the stromal cells and the hematopoietic cells can be performed simply after the collection of the bone marrow cells without further separation. Furthermore, co-culture can be performed by separating components such as stromal cells, hematopoietic cells and other cell populations from collected bone marrow and combining them with the hematopoietic cells and stromal cells which are not from the individual from which the bone marrow is cllected. Furthermore, after stromal cells are cultured to grow to the stromal cells, hematopoietic cells can be added to perform co-culture with the hematopoietic stem cells. At this time, cell stimulating factors can added to the culture system of stromal cells to more effectively support proliferation and survival. Concrete examples of the cell stimulating factor include a growth factor which is typically a cytokine such as SCF (stem cell factor), IL-3 (interleukin 3), GM-CSF (granulocyte/macrophage colony-stimulating factor), IL-6 (interleukin 6), TPO (thrombopoietin), G-CSF (granulocyte colony-stimulating factor), TGF-b (transforming growth factor-b), MIP-1a (Davatelis, G., J. Exp. Med. 167: 1939, 1988); a differentiation and proliferation control factor such as hematopoietic hormones such as EPO (erythropoietin), chemokine, Wnt gene product, and notch ligand; and a development control factor.

In addition, when the polypeptide of the present invention affects hematopoietic stem cells or hematopoietic progenitor cells to show an activity to support survival or proliferation thereof, in other words, the polypeptide has an activity to support survival or proliferation of hematopoietic stem cells or hematopoietic progenitor cells if the hematopoietic stem cells or the hematopoietic progenitor cells are cultured in the presence of the polypeptide, the proliferation and the survival of the hematopoietic stem cells or the hematopoietic progenitor cells can be retained by allowing the recombinant polypeptide of the present invention alone or in combination with the cell stimulating factors to affect hematopoietic stem cells or hematopoietic progenitor cells, without stromal cells. Examples of the cell stimulating factors used in this case are above described cell stimulating factors and the like.

Medium used for the culture is not specially restricted as long as the proliferation or the survival of the hematopoietic stem cells or the hematopoietic progenitor cells is not harmed. Preferable media are, for example, MEM-α medium (GIBCO BRL), SF-02 medium (Sanko Junyaku), Opti-MEM medium (GIBCO BRL), IMDM medium (GIBCO BRL), and PRMI1640 medium (GIBCO BRL). A culture temperature is usually ranging from 25 to 39°C, and preferably ranging from 33 to 39°C. Examples of

additives to the medium are fetal bovine serum, human serum, horse serum, insulin, transferrin, lactoferrin, ethanolamine, sodium selenite, monothiolglycerol, 2-mercaptoethanol, bovine serum albumin, sodium pyruvate, polyethylene glycol, a variety of vitamins, and a variety of amino acids. A concentration of CO_2 is usually ranging from four to six percent, and preferably five percent.

Since the hematopoietic stem cells can differentiate into all the hematopoietic cell lineages, the hematopoietic stem cells can be amplified and differentiated into a specific cell type *in vitro*, and then the specific cells can be transplanted. For example, when erythrocytes are necessary, after the cultivation of the patient's stem cells to amplify them, the hematopoietic cells whose main component is the erythrocyte can be artificially produced using an erythrocyte differentiation induction-promoting factor such as EPO.

The hematopoietic stem cells or the hematopoietic progenitor cells cultured using the polypeptides of the present invention can be used as a graft for blood cell transplantation replacing the conventional bone marrow transplantation or cord blood transplantation. Transplantation of the hematopoietic stem cells is superior to the conventional blood cell transplantation therapy, since the engraftment can last semipermanently.

The transplantation of the hematopoietic stem cells can be employed as therapy for a variety of diseases in addition to combination therapy with total body X-ray irradiation therapy or advanced chemotherapy for leukemia. For example, when therapy accompanied with myelosuppression as an adverse reaction, such as chemotherapy, radiation therapy, and the like is performed for the patient with solid cancer, the patient can get benefit of early recovery and stronger chemotherapy than the conventional one can be performed to improve the therapeutic effect of the chemotherapy by collecting the bone marrow before the therapy, allowing the hematopoietic stem cells or the hematopoietic progenitor cells to be amplified *in vitro* and returning the amplified cells to the patient after the therapy. In addition, by allowing the hematopoietic stem cells or the hematopoietic progenitor cells obtained according to the present invention to be differentiated into a variety of hematopoietic cells and transplanting these cells into a patient with hypoplasia of a given hematopoietic cells, the patient's deficient status can be improved. In addition, this therapy can improve dyshemopoietic anemia to develop anemia such as aplastic anemia caused by bone marrow hypoplasia. Furthermore, examples of diseases in which the transplantation of the hematopoietic stem cells according to the present invention is effective include immunodeficiency syndrome

such as chronic granulomatous disease, duplicated immunodeficiency syndrome, agammaglobulinemia, Wiskott-Aldrich syndrome, acquired immunodeficiency syndrome (AIDS), and the like, thalassemia, hemolytic anemia due to an enzyme defect, congenital anemia such as sicklemia, Gaucher's disease, lysosomal storage disease such as mucopolysaccharidosis, adrenoleukodegeneracy, a variety of cancers and tumors, and the like.

Transplantation of the hematopoietic stem cells may be performed in the same manner as the conventional bone marrow transplantation or cord blood transplantation other than the differences of the cells used.

The source of the hematopoietic stem cells which may be used for the above described hematopoietic stem cell transplantation is not restricted to the bone marrow, and the above described fetal liver, the fetal bone marrow, the peripheral blood, the peripheral blood with stem cells mobilized by administration of cytokines and/or antitumor drugs, the cord blood, and the like may be used.

The graft may be a composition including buffer solution and the like in addition to the hematopoietic stem cells and the hematopoietic progenitor cells produced by the method according to the present invention.

The hematopoietic stem cells or the hematopoietic progenitor cells produced according to the present

invention may be used for *ex vivo* gene therapy. Because of the low frequency of recombination of target genes to the chromosome because the stem cells are in the resting state, differentiation of the stem cells during the culture period, and the like, the gene therapy to the hematopoietic stem cells has been hard to be established. However, the present invention can amplify the stem cells without differentiation, so that efficacy of gene transfer is expected to be remarkably improved. In this gene therapy, a foreign gene (a gene for therapy) is transferred into the hematopoietic stem cells or the hematopoietic progenitor cells, and then the obtained gene-transferred cells are used. The foreign gene to be transferred is appropriately selected according to disease. Examples of diseases in which the target cells of the gene therapy are the hematopoietic cells include immunodeficiency syndrome such as chronic granulomatous disease, duplicated immunodeficiency syndrome, agammaglobulinemia, Wiskott-Aldrich syndrome, acquired immunodeficiency syndrome (AIDS), and the like, thalassemia, hemolytic anemia due to an enzyme defect, congenital anemia such as sicklemia, Gaucher's disease, lysosomal storage disease such as mucopolysaccharidosis, adrenoleukodegeneracy, a variety of cancers and tumors, and the like.

A usual method used for transfer of a gene into animal cells is employed for the transfer of the gene

for the therapy into the hematopoietic stem cells or the hematopoietic progenitor cells. Examples include a method using a vector for animal cells derived from virus utilized for a gene therapy such as retrovirus vectors such as Moloney mouse leukemia virus, adenovirus vectors, adeno-associated virus (AAV) vectors, herpes simplex virus vectors, and HIV vectors (with respect to a vector for gene therapy, see Verma, I.M., Nature, 389: 239, 1997); calcium phosphate transfection, DEAE-dextran transfection, electroporation, the liposome method, the lipofection method, the microinjection method, and the like. Among them, the method using the retrovirus vector, the adeno-associated virus vector, or the HIV vector is preferable, since permanent expression of a gene is expected due to insertion into the chromosome DNA of a target cell.

For example, the adeno-associated virus (AAV) vector can be prepared as follows. First, a vector plasmid in which a gene for therapy is inserted into ITR (inverted terminal repeat) at both ends of wild-type adeno-associated virus DNA and a helper plasmid for supplementing virus proteins are transfected into 293 cell line. Next, adenovirus as helper virus is infected, so that virus particles including the AAV vector are produced. Alternatively, instead of adenovirus, a plasmid which expresses adenovirus gene having helper function may be transfected. The hematopoietic stem

cells or the hematopoietic progenitor cells are infected with the obtained virus particles. Preferably, appropriate promoter, enhancer, insulator and the like are inserted into the upstream region of the target gene in the vector DNA, so that the expression of the gene is regulated. When a marker gene such as a drug resistant gene is used in addition to the gene for therapy, cells into which the gene for therapy are transferred are easily selected. The gene for therapy may be a sense gene or an antisense gene.

A composition for gene therapy may include a buffer solution and a novel active ingredient and the like in addition to the hematopoietic stem cells or the hematopoietic progenitor cells by the method according to the present invention.

A vector for gene therapy can be produced by incorporating the DNA of the present invention in an expression vector using a usual method. This vector for gene therapy is useful to treat diseases which need survival and proliferation of the human hematopoietic stem cells. That is, the vector for gene therapy is transferred into the hematopoietic stem cells and the cells are cultured *in vitro*, so that the hematopoietic stem cells or the hematopoietic progenitor cells can proliferate dominatingly. The proliferation of hematopoietic stem cells *in vivo* can be caused by returning these hematopoietic stem cells thus treated.

The proliferation of hematopoietic stem cells *in vivo* can significantly promoted by introducing this vector for gene therapy into the body. Alternatively, the bone marrow cells derived from a patient are cultured as it is and this vector for gene therapy is transferred thereto, so that the hematopoietic stem cells or the hematopoietic progenitor cells can be proliferated in a culture system. Alternatively, this vector for gene therapy is transferred into the stromal cells and mesenchaymal stem cells obtained by isolating and culturing stromal cells from the bone marrow, so that the activity to support the hematopoietic stem cells can be added or increased.

As shown in Examples, since it is possible that by introducing the DNA of the present invention into the stromal cells without the activity to support the hematopoietic stem cells, this activity can be imparted, stromal cells having the activity to support the hematopoietic stem cells can be prepared by gene transfer to stromal cells derived from human or mouse. The stromal cells expressing the DNA of the present invention and the hematopoietic stem cells or the hematopoietic progenitor cells are co-cultured, so that the hematopoietic stem cells or the hematopoietic progenitor cells can survive and proliferate so as to be useful for a variety treatment.

Since the hematopoietic stem cells or the

hematopoietic progenitor cells can survive and proliferate by expression of the DNA of the present invention in the stromal cell, an activity to support the hematopoietic stem cells of the stromal cells can be determined using the expression of the DNA of the present invention as an index. The expression of the DNA of the present invention in the stromal cells can be confirmed using an antibody against a polypeptide encoded by the DNA of the present invention. Also, PCR primers can be prepared based on nucleotide sequences, and RNA is prepared from the stromal cells of interest, and RT-PCR is performed, so that the expression of the DNA of the present invention can be confirmed. The antibody will be described below.

The pharmaceutical composition of the present invention can be administered to human. The pharmaceutical composition having an activity to proliferate or to support the human hematopoietic stem cells or the hematopoietic progenitor cells can be produced by mixing medically acceptable diluent, stabilizer, carrier, and/or other additives with the polypeptides of the present invention. At this time, in order to increase the stability of the protein *in vivo*, the polypeptides of the present invention may be modified by a modifying agent. Examples of the modifying agent include polyethylene glycol (PEG), dextran, poly(N-vinyl-pyrrolidone), polypropylene glycol

homopolymer, polypropylene oxide/ethylene oxide copolymer, polyoxyethylated polyol, polyvinyl alcohol, and the like. The modification of the protein with PEG can be performed by, for example, a method in which activated ester derivatives of PEG is reacted with the protein, a method in which aldehyde derivatives at the terminal portion of PEG is reacted with the protein in the presence of a reducing agent, and the like. Japanese Patent Application Laid-Open No. 10-510980 discloses such protein modification in detail.

When the pharmaceutical composition of the present invention is administered to human, recovery from hematological suppression due to an adverse drug reaction of carcinostatics; early recovery of hematopoietic cells at bone marrow transplantation, peripheral blood stem cell transplantation, or cord blood transplantation; and recovery of hematopoietic function at pancytopenia such as aplastic anemia (AA) and myelodysplastic syndrome (MDS) are expected.

The antibodies of the present invention react specifically to the above described polypeptides of the present invention. The antibodies of the present invention may be monoclonal antibodies or polyclonal antibodies as long as they react specifically to the above described polypeptides.

The antibodies of the present invention can be prepared according to usual methods. For example, the

antibodies can be prepared either *in vivo* method in which animals are additionally immunized by an antigen together with adjuvant once or several times at an interval of several weeks or *in vitro* method in which immune cells are isolated and sensitized in an appropriate culture system. Examples of immune cells which can produce the antibodies of the present invention include splenic cells, tonsillar cells, lymph gland cells, and the like.

The whole polypeptide according to the present invention is not necessarily used as an antigen. A part of this polypeptide may be used as an antigen. When the antigen is a short peptide, particularly, a peptide made of about 20 amino acid residues, it may be used by binding it to a carrier protein having high antigenicity such as keyhole lympet hemocyanin or bovine serum albumin using chemical modification and the like. Alternatively, the antigen may be used by covalently binding it to peptide having branching skeleton such as lysine core MAP peptide instead of the carrier protein (Posnett *et al.*, *J. Biol. Chem.*, *263*, 1719-1725, 1988; Lu *et al.*, *Mol. Immunol.*, *28*, 623-630, 1991; Briand *et al.*, *J. Immunol. Methods*, *156*, 255-265, 1992).

Examples of adjuvant include Freund's complete adjuvant, Freund's incomplete adjuvant, aluminum hydroxide gel, and the like. Antigen-given animals are, for example, mouse, rat, rabbit, sheep, goat, chicken,

bovine, horse, guinea pig, hamster, and the like. The blood is collected from these animals and the serum is separated. Then, immunoglobulin is purified from the serum using an ammonium sulfate precipitation method, anion exchange chromatography, protein A chromatography, or protein G chromatography to obtain polyclonal antibodies.

With respect to chicken, antibodies can be purified from an egg. Monoclonal antibodies can be prepared by purification from supernatant of culture of hybridoma cells which are made by fusion of the immune cells sensitized *in vitro*, or immune cells from the above described animals with parent cells capable of cultivation, or ascites from animals which received intraperitoneal administration of hybridoma cells. Examples of parent cells include X63, NS-1, P3U1, X63.653, SP2/O, Y3, SK0-007, GM1500, UC729-6, HM2.0, NP4-1 cell lines, and the like. Preparation may be performed by cultivating the immortalized antibody-forming cells obtained by sensitization *in* vitro, or infection of a proper virus such as EB virus to the immune cells of the above described animals.

In addition to these cell engineering methods, the antibodies can be obtained using gene engineering methods. For example, the antibody gene obtained from the *in vitro* sensitized cells or immune cells derived from the above described animals is amplified by PCR

(polymerase chain reaction) and isolated, and the amplified genes are transferred into microorganisms such as *E. coli* to produce the antibodies. Alternatively, the antibodies may be expressed on surfaces of phages as fused proteins.

By measuring polypeptides *in vivo* using the antibodies of the present invention, the relationship between the polypeptides and pathological status of a variety of diseases can be clarified. Moreover, the antibodies can be used for diagnosis and treatment of diseases, and efficient affinity purification of the polypeptides.

The present invention provides polypeptides having an activity to support survival or proliferation of hematopoietic stem cells or hematopoietic progenitor cells by effecting thereon, or an activity to impart an activity to support the hematopoietic stem cells to stromal cells by effecting thereon, and also provides DNAs encoding thereof. The polypeptides of the present invention can efficiently maintain the proliferation or the survival of the hematopoietic stem cells or the hematopoietic progenitor cells.

Examples

Hereafter, the present invention will be described in detail by reference to examples.

Example 1 Preparation of fragment of gene which is specifically expressed in hematopoietic stem cell-supporting cells

(I) Preparation of stromal cell line derived from mouse AGM

(1) Isolation of AGM region from fetal mouse

C3H/HeNSLc mice of both genders (purchased from Japan SLC INC.) were kept under a SPF (specific pathogen-free) environment. One or two female mice and one male mouse were placed in the same cage over a night. In the next morning, the female mice in which the existence of a vaginal plug was observed were transferred to other cages and kept. The day when the existence of the vaginal plug was observed was defined to be the 0.5th day of pregnancy. On the 10.5th day of the pregnancy, after mouse was sacrificed by cervical dislocation, fetuses were extirpated. Isolation of AGM regions was performed according to the method by Godin *et al.* (Godin, I., *Proc. Natl. Acad. Sci. U.S.A.*, *92*: 773-777, 1995) and the method by Medvinsky *et al.* (Medvinsky, A.L., *Blood*, *87*: 557-565, 1996). The fetuses were placed in a culture dishes to which PBS(-) (phosphate buffered saline) (produced by Nissui Seiyaku) was added in a volume just sufficient to cover the fetuses. After the AGM regions were carefully excised so as not to include other regions under a stereoscopic microscope, they were put in another 24-well culture

dish (Nunc).

(2) Establishment of cell lines derived from AGM

One drop of MEM medium (Sigma) containing 10% FCS (Hyclone) was added to the AGM regions in the 24-well culture dish (Nunc), and AGM regions were cultured in an incubator overnight. The culture was performed in the MEM medium (Sigma) containing 10% FCS (Hyclone) at 37°C, in an atmosphere of 5% CO_2, and at a humidity of 100%. When the cells of the AGM regions adhered to the culture dish due to overnight cultivation, two milliliters of MEM medium containing 10% FCS was further added. Stromal cells began to appear around the AGM region tissue fragment after the continuous cultivation. After one-week cultivation, adhesive cells were separated by trypsin treatment (0.05% trypsin in PBS containing 0.53 mM EDTA (Gibco BRL) at 37°C for three to five minutes). The stromal cells were then washed twice with the medium, and seeded on 6-well culture dish (Nunc). On the next day, the cells which did not adhere to the culture dish and the medium were removed, and then, fresh medium was added. Two weeks after transfer to the 6-well culture dish, cells were γ-ray-irradiated at 900 Rad to eliminate endogenous hematopoietic cells. An attempt of the direct cell cloning by limiting dilution from this culture system was made, but no cell proliferation was observed and the cloning ended in failure. Then, after the number of seeded cells in one well was increased and

cells were adapted so as to be able to proliferate from a small number of cells, the cells were cloned by limiting dilution.

Specifically, the AGM was extirpated and cultured in the same manner as described above. The culture system two weeks after the γ-ray radiation was trypsinized (0.05% trypsin in PBS containing 0.53 mM EDTA at 37°C for three to five minutes) to suspend the cells, and the cells were seeded in a 24-well culture dish at 50 to 100 cells/well. After the culture was continued for three weeks, the cells were seeded in a 96-well culture dish (Nunc) by means of limiting dilution, at 0.3 cells/well. The cells which were grown from the well in which only one cell was seeded were allowed to enlarge culture. As a result, the cells were successfully cloned to obtain fibroblast-like cells and cobble stone-like cells.

A CD34-positive cell fraction derived from the human cord blood was co-cultured with the fibroblast-like cells for two weeks to examine the presence of colony-forming cells during the culture. Colony-forming cells could not be found in the co-culture system with the fibroblast-like cells. Then, the similar examination was performed for seven cell clones showing the cobble-stone-like form. Three clones having an activity to support proliferation of human hematopoietic stem cells were obtained and were named AGM-s1, AGM-s2, and AGM-s3.

(II) Preparation of hematopoietic stem cells from mouse

bone marrow

Bone marrow was collected from a femur of C57BL/6-Ly5.1 pep (eight- to ten-week age, and male) (the gift from Professor K. Nakauchi, University of Tsukuba), and suspended in PBS. After the mouse bone marrow mononuclear cells were concentrated by specific gravity centrifugation according to the usual method (S. Kouzu, Fundamental techniques for immunology, YODOSHA, 1995), the cells were suspended in staining buffer (PBS containing 5% FCS and 0.05% NaN_3), and a hematopoietic stem cell fraction was obtained as follows (Osawa, M. et al., Science 273: 242-245, 1996).

An FITC-conjugated anti-CD34 antibody, a phycoerythrin-conjugated anti-Sca-1 antibody, an allophycocyanin anti-c-Kit antibody (all purchased from Pharmingen) and six biotylated anti-differentiation antigen antibodies (CD45R, CD4, CD8, Gr-1, Ter119, and CD11c, all purchased from Pharmingen) as molecular markers (Lin), were added to a suspension of the bone marrow mononuclear cells and incubated for 20 min on ice to cause reaction. After the cells were washed twice with staining buffer, CD34-negative, Sca-1-positive, c-Kit-positive, and Lin-negative cells were isolated on a cell sorter (FACS Vantage, Becton Dickinson).

(III) Subcloning of mouse stromal cell line and determination of activity to support hematopoietic stem cells of a variety of cell lines

(1) Subcloning of mouse stromal cell line

1) Isolation of AGM-s3 subclone

Stromal cell line AGM-s3 derived from AGM, which was subcultured in MEMα medium (GIBCO BRL) including inactivated 10% FCS (bovine fetal serum, Hyclone), was suspended in PBS containing 5% FCS (PBS-FCS). Clone sorting was performed in a 96-well culture dish (Falcon) at one cell/well using a cell sorter (FACS Vantage; Becton Dickinson). Among cells in the 96 wells, cultures of the cells which grew were expanded, so that thirteen kinds of AGM-s3 subclones were obtained. The activity to support the hematopoietic cells of these AGM-s3 subclones were examined.

2) Isolation of human cord blood CD34-positive stem cell

The human cord blood was collected at normal delivery according to the criteria approved by Ethics committee of Kirin Beer Iyaku Tansaku Kenkyusho. The cord blood was collected using a heparin-added syringe so as not to coagulate. The heparin treated cord blood was overlaid on Lymphoprep (NYCOMED PHARMA), and mononuclear cells were separated by specific gravity centrifugation (at 400G, at room temperature, and for 30 minutes). Erythrocytes contaminated in the mononuclear cell fraction were lyzed by treatment with an ammonium chloride buffer solution (0.83% NH_4Cl-Tris HCl, 20 mM, pH 6.8) at room temperature for two minutes. After the mononuclear cells were washed with PBS-FCS, ten

milligrams of human IgG was added thereto and the mixture was allowed to stand on ice for ten minutes. Then, the cells were further washed with PBS-FCS. Biotinylated antibodies against the antigens specific to the human differentiated blood cells, that is, the antibodies against CD2, CD11c (purified from ATCC hybridoma), CD19 (Pharmingen), CD15, and CD41 (Leinco Technologies Inc.), and Glycophorin A (Cosmo Bio) were added thereto, and the mixture was allowed to stand on ice for 20 min. After washing with PBS-FCS, the cells were suspended in one milliliter of PBS containing 5% FCS, 10 mM EDTA, and 0.05% NaN_3 (PBS-FCS-EDTA-NaN_3). Streptavidin-bound magnetic beads (BioMag. Per Septive Diagnostics) were added thereto, and the mixture was allowed to stand on ice for 40 min. The differentiated blood cells which expressed differentiation antigens were removed using a magnetic separator (Dynal MPC-1 Dynal). An FITC-labeled anti-CD34 antibody (Immunotech S.A., Marseilles, France) was added to the remaining differentiated blood cell antigen-negative cell fraction. After incubation on ice for 20 min., a CD34-positive fraction was recovered using a cell sorter. This cell population was defined as a hematopoietic stem cell population derived from the human cord blood.

3) Co-culture of the human hematopoietic stem cells and AGM-s3 subclone

After 13 kinds of AGM-s3 subclones and stromal cell

line MS-5 derived from the mouse bone marrow were each seeded in a 24-well culture dish (Falcon) at 1×10^4 cells/well, and cells were cultured in one milliliter of MEMα medium containing 10% FCS and allowed to grow until the cells covered all over the bottom surfaces of the wells. CD34-positive hematopoietic stem cells derived from the human cord blood were placed on the above described stromal cells at 500 cells/well, and co-cultured in one milliliter of MEMα medium containing 10% FCS. One week after the start of the co-culture, one milliliter of the same medium was further added. Two weeks after the start of the co-culture, the stromal cells and the human blood cells were trypsinized (0.05% trypsin in PBS containing 0.5 mM EDTA (GIBCO BRL) at 37°C; standing for two to five min.) to simultaneously separate them from the culture dish. An activity to support the hematopoietic stem cells was determined with a clonogenic assay.

4) Assessment of proliferation statuses of the hematopoietic stem cells and hematopoietic progenitor cells by clonogenic assay

The cells which proliferated in the above described co-culture system were appropriately diluted, and subjected to one milliliter of methylcellulose culture system to be analyzed. The analysis using the methylcellulose culture system was performed using a 6-well culture dish (Falcon) in MethoCult H4230 (Stem Cell

Technologies Inc.) to which 10 ng/ml of human SCF, human IL-3, human IL-6, human G-CSF, and human TPO, and 2 IU/ml of EPO were added. All of a variety of the above described hematopoietic factors were recombinants and pure. After two-week culture, developed colonies were observed under a microscope to count numbers of CFU-GM (granulocyte-macrophage colony-forming unit), BFU-E (erythroid burst forming unit), and CFU-E mix (erythrocyte mixed colony-forming unit).

Fig. 1 shows the result of two-week co-culture of the CD34-positive hematopoietic stem cells and the AGM-s3 subclone A9, A7, or D11. As a result of the co-culture, A9 and D11 subclones among 13 kinds of AGM-s3 subclones supported proliferation of all three series of CFU-GM, BFU-E, and CFU-E mix. Especially, although BFU-E and CFU-E mix, that is, the progenitor cells of erythrocytes were hardly to be supported in usual, their proliferations were observed in the co-culture system with A9 or D11 cells. The results showed that proliferation or maintenance of the hematopoietic stem cells or the hematopoietic progenitor cells occurred in the co-culture with A9 or D11 cells and the progenitor cells of the erythrocyte were continuously supplied. In contrast, although cellular morphology of A7 was similar to that of A9, A7 did not support CFU-GM, BFU-E, and CFU-E mix.

5) Comparison of an activity to support the human

hematopoietic stem cells between A9 and a stromal cell line OP9 derived from mouse fetus

Comparison of an activity to support the CD34-positive hematopoietic stem cells derived from the human cord blood between AGM-s3 subclones A9 and A7, and a stromal cell line OP9 derived from mouse fetus (RCB1124, the Cell Development Bank of RIKEN) were performed with CFU-GM, BFU-E, CFU-E and CFU-E mix as indexes, using the above described determination system. Fig. 2 shows the result of the two-week co-culture. In the A7 cell culture system, CFU-GM, BFU-E, and CFU-E were significantly decreased and CFU-E mix was completely disappeared. In contrast, with OP9 cells, a variety of blood cell progenitor cells including CFU-E mix were supported, although the supporting ability was less than that of A9 cells. Therefore, it has been found that OP9 cells possess the activity to support the hematopoietic stem cells.

(2) Assessment of activity to support the hematopoietic stem cells in a variety of cell lines

The above described stromal cell lines (AGM-s3-A9, AGM-s3-A7, and AGM-s3-G1), 3T3Swiss (ATCC), OP9, and NIH3T3 (ATCC) were seeded in a 24-well culture dish (Falcon) at 5×10^4 cells/well. The cell lines were cultured in MEMα medium (GIBCO BRL) containing inactivated 10% FCS (bovine fetal serum, Hyclone) for one day and allowed to proliferate until the cells

covered all over the bottom surfaces of the wells. Then, the medium was replaced to one milliliter of fresh medium, thirty cells of the mouse hematopoietic stem cells (derived from C57BL/6-Ly5.1) obtained in the above (II) were placed on this cell layer, and co-culture was started.

On seventh day of the cultivation, the cells were trypsinized (0.05% trypsin in PBS containing 0.5 mM EDTA (GIBCO BRL) at 37°C for two to five minutes) to separate and recover all the cells on the culture dish. The recovered whole cells of each cell line and 200,000 cells of whole bone marrow cells (derived from C57BL/6-Ly5.2 mouse, Charles River) were transplanted into C57BL/6-Ly5.2 mice (eight weeks age and male, Charles River) irradiated with X-ray at 8.5 Gy through the tail vein. After the transplantation, peripheral blood was collected from orbit at intervals, and the ratio in number of cells derived from the C57BL/6-Ly5.1 prep mouse was determined with FACS. The peripheral blood was analyzed according to the usual method (S. Kouzu, Fundamental techniques for immunology, YODOSHA, 1995). Three hundreds and fifty μL of distilled water was added to 50 μL of the peripheral blood, and the mixture was allowed to stand for 30 seconds so as to lyze the erythrocytes. Then, PBS at twice concentrations was added and the mixture was centrifuged to recover white blood cells. After the cells were washed once using the

staining buffer (PBS containing 5% FCS and 0.05% NaN_3), anti-CD16 antibody, anti-Ly5.1 (CD45.1) antibody labeled with FITC, anti-Gr-1 and anti-CD11c antibodies labeled with phycoerythrin, and anti-CD45R (B220) and anti-CD90 (Thy1) antibodies labeled with allophycocyanin (all of these were purchased from Pharmingen) were added. After these cells were allowed to stand for reaction in the ice bath for 30 minites, they were washed with the staining buffer and FACS analysis was performed.

Change in the number of cells capable of reconstitution during the hematopoietic stem cell culture was determined by calculating the proportions of Ly5.1-positive cells in the Gr-1- or CD11c-positive cells (myeloid cells) and Ly5.1-positive cells in the CD90- or CD45R-positive cells (lymphoid cells) in the peripheral blood at intervals after transplantation.

Fig. 3 shows the results. When the cells were co-cultured with AGM-s3-A9 cells, OP9 cells, or 3T3Swiss cells, high chimerism of donor cells were maintained after the transplantation. Therefore, these stromal cells were considered to have a high activity to support the hematopoietic stem cells. In contrast, when the cells were co-cultured with AGM-s3-A7 cells, AGM-s3-G1 cells, or NIH3T3 cells, high chimerism derived from the transplanted cells was not observed. Therefore, these stromal cells were low in an activity to support the hematopoietic stem cells or the hematopoietic progenitor

cells.

(IV) Identification of sequences of genes which specifically express in hematopoietic stem cell-supporting cells

AGM-s3-A9 cells, AGM-s3-A7 cells and OP9 cells were each dissolved in 20 mL of ISOGEN (Nippon gene, Japan) and total RNAs were prepared according to the attachment. Messenger RNAs were prepared from one milligram of the total RNAs according to the protocol of the mRNA purification kit (Amersham Pharmacia, U.S.A.). cDNAs were synthesized from the mRNAs and cDNA libraries (hereinafter, also called as AGM-s3-A9 cDNA, AGM-s3-A7 cDNA and OP9 cDNA, respectively) were constructed using pSPORT1 (GIBCO Lifetech, U.S.A.). A clone harboring a cDNA fragment which highly expresses specifically to AGM-s3-A9 cells or OP9 cells compared with AGM-s3-A7 cells was obtained from the libraries with SBH method (Hyseq, U.S.A.). A nucleotide sequence of the obtained clone was determined using ABI377 DNA sequencer (Perkin Elmer, U.S.A.).

As a result, it has been found that expression of genes comprising nucleotide sequences shown in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, and SEQ ID NO:7, or parts thereof in AGM-s3-A9 or OP9 cells is higher than that in AGM-s3-A7 cells. These genes were named as SCR-2, SCR-3, SCR-4, SCR-5, SCR-6, SCR-7 and SCR-8, respectively.

Example 2 Cloning of SCR-2 and activity determination

By searching GenBank database for the nucleotide sequence shown in SEQ ID NO: 1 with BLAST, it has been found that SCR-2 is the same gene as a mouse gene, *Mus musculus* glypican-1 (GPC-1) of an accession number AF185613. The nuclotide sequence of ORF (Open Reading Frame) of SCR-2 and the amino acid sequence deduced from the nucleotide sequence are shown in SEQ ID NO: 8. Only the amino acid sequence is shown in SEQ ID NO: 9.

The human nucleotide sequence of GPC-1 is recorded in GenBank database under an accession number AX020122. The nucleotide sequence of ORF of AX020122 and the amino acid sequence deduced from the nucleotide sequence are shown in SEQ ID NO: 10. Only the amino acid sequence is shown in SEQ ID NO: 11.

Determination of the activity to support the hematopoietic stem cells or hematopoietic progenitor cells was performed as follows.

(1) Construction of retrovirus vector for expression of mouse SCR-2

Based on the nucleotide sequence of SCR-2 ORF, SCR-2Fsal1 and SCR-2Reco primers having the following nucleotide sequences were prepared, and PCR was performed using OP9 cDNA as a template.

SCR-2Fsal

CCGGTCGACCACCatggaactccggacccgaggctgg (SEQ ID NO: 30)

SCR-2Reco

CCGAATTCttaccgccacctgggcctggctgc (SEQ ID NO: 31)

An amplified fragment was digested with restriction enzymes *Eco*RI and *Sal*I. After electrophoresis, a DNA fragment was purified using JETSORB (Genomed, Germany). The purified DNA fragment was ligated with pMX-IRES-GFP vector digested with *Eco*RI and *Xho*I (gift form Professor T. Kitamura, TOKYO UNIV. INST. OF MEDICAL SCIENCE, Japan). The pMX-IRES-GFP vector is a plasmid obtained by inserting sequences encoding IRES (Internal Ribosome Entry Site) and GFP (Green Fluorescence Protein) into the retrovirus vector pMX. IRES enables ribosome to access to the middle of the mRNA. Therefore, two genes can be expressed from one mRNA by ligation of upward and downward genes separated by IRES in one transcription unit during the construction of an expression vector. With respect to the above-described plasmid, SCR-2 cDNA was inserted in the upward site and GFP (Green Fluorescence Protein) was inserted in the downward site. Thus, the expression of SCR-2 could be monitored by detecting the expression status of GFP using FACS.

The obtained recombinant vector was introduced into *E. coli* DH5α, and was seeded on LB agar medium containing 100 μg/ml of ampicillin, so that independent colonies were formed. After the isolated colony was cultured in 100 mL of LB medium containing 100 μg/ml of ampicillin, plasmid was purified using QIAGENtip100

(QIAGEN, U.S.A.). The sequence of the inserted gene was determined using a conventional method, so that the sequence was confirmed to be identical to the nucleotide sequence of SCR-2 ORF.

(2) Preparation of stromal cells highly expressing SCR-2

BOSC23 cells were seeded on a collagen type I-coated 60-mm dish (Asahi technoglass) at 2 × 10^6 cells/dish, and cultured in DMEM medium (GIBCO BRL) containing 10% FCS at 37°C, under an atmosphere of 5% CO_2, and at a humidity of 100%. Twelve to 18 hours after the start of the culture, the medium was replaced by two milliliters of OPTI MEM medium (GIBCO BRL).

About 3 μg of plasmid obtained by inserting SCR-2 into the above described pMX-IRES-GFP was added to 18 μl of LIPOFECTAMINE Reagent (GIBCO BRL) diluted with 100 μl of OPTI MEM medium, and the mixture was allowed to stand at room temperature for 30 min. The prepared DNA solution was added to the prepared BOSC23 cell culture solution. After about five hours, two milliliters of DMEM medium containing 20% FCS (GIBCO BRL) was added.

After about 24 hours, the medium was replaced by 4 ml of DMEM containing 10% FCS. Further, after about 48 hours, the culture medium was harvested. After the culture medium was filtrated through 0.45-μm filter, the filtrate was centrifuged at 1,200g for 16 hours and the supernatant was removed to obtain the virus precipitation.

AGM-s3-A7 or AGM-s3-A9 cells were cultured in one milliliter of MEMα medium containing 10% FCS (GIBCO BRL) on a 24-well culture dish (FALCON) at 1×10^4 cells/well. After 12 to 18 hours, the virus precipitation was suspended in one milliliter of MEMα medium containing 10% FCS, and the stromal cell culture medium was replaced by the virus suspension. Next, POLYBRENE (Sigma, SEQUA-BRENE) was added to be 10 μg/ml. After the culture dish was centrifuged at 700g for 45 minutes, the cells were cultured at 37°C, under an atmosphere of 5% CO_2, and at a humidity of 100%. After 48 hours, the medium was replaced by one milliliter of MEMα medium containing 10% FCS. After 24 hours, the cells were subcultured on a 6-well culture dish (FALCON) and cultured in three milliliters of MEMα medium containing 10% FCS. Forty-eight hours after the subculturing, GFP expression in the stromal cells was detected using a cell sorter (FACSVantage, Becton Dickinson) to indirectly confirm that not less than 80% of cells expressed SCR-2.

Also, the same procedures were repeated by using pMX-IRES-GFP vector instead of the plasmid obtained by inserting SCR-2 into pMX-IRES-GFP to prepare stromal cells into which a control vector was introduced.

(3) Co-culture of human hematopoietic stem cells and stromal cells highly expressing SCR-2, and determination of proliferation statuses of hematopoietic stem cells

and hematopoietic progenitor cells by clonogenic assay

In the same manner as described in (III) (1) 3) to 4) of Example 1, AGM-s3-A9 or AGM-s3-A7 cells in which SCR-2 was highly expressed through retrovirus, AGM-s3-A9 or AGM-s3-A7 cells into which a control vector was introduced, or AGM-s3-A9 or AGM-s3-A7 cells were co-cultured with CD34-positive hematopoietic stem cells derived from human cord blood, and proliferation statuses of hematopoietic stem cells and hematopoietic progenitor cells are determined.

Fig. 4 shows results when the CD34-positive hematopoietic stem cells were co-cultured with AGM-S3-A9 cells in which SCR-2 was highly expressed (A9/SCR-2), AGM-S3-A9 cells into which a control vector was introduced (A9/pMXIG) or AGM-S3-A9 cells (A9) for two weeks. Also, Fig. 5 shows results when the CD34-positive hematopoietic stem cells were co-cultured with AGM-S3-A7 cells in which SCR-2 was highly expressed, AGM-S3-A7 cells into which a control vector was introduced or AGM-S3-A7 cells for two weeks. As a result, by the co-culture with AGM-S3-A9 cells in which SCR-2 was highly expressed or AGM-S3-A7 cells in which SCR-2 was highly expressed, increases of BFU-E and CFU-C were observed. Therefore, it has been revealed that the activity to support hematopoietic stem cells or hematopoietic progenitor cells, of AGM-S3-A9 or AGM-S3-A7 increases by allowing SCR-2 to be highly expressed.

From the results, it has been revealed that a gene product of SCR-2 has an activity to support survival or proliferation of hematopoietic stem cells or hematopoietic progenitor cells or an activity to affect stromal cells to enhance a hematopoietic cell-supporting activity of the stromal cells or impart the activity to the stromal cells.

Example 3 Cloning of SCR-3 and activity determination

By searching GenBank database for the nucleotide sequence shown in SEQ ID NO: 2 with BLAST, it has been found that SCR-3 is the same gene as mouse genes, *Mus musculus* chemokine MMRP2 mRNA of an accession number U15209, *Mus musculus* C10-like chemokine mRNA of U19482 and mouse macrophage inflammatory protein-1gamma mRNA of U49513. The nuclotide sequence of SCR-3 ORF and the amino acid sequence deduced from the nucleotide sequence are shown in SEQ ID NO: 12. Only the amino acid sequence is shown in SEQ ID NO: 13.

Determination of the activity of SCR-3 to support the hematopoietic stem cells or hematopoietic progenitor cells was performed as follows.

(1) Construction of retrovirus vector for expression of mouse SCR-3

Based on the nucleotide sequence of SCR-3 ORF, SCR-3FxhoI and SCR-3Reco primers having the following nucleotide sequences were prepared, and PCR was

performed using AGM-s3-A9 cDNA as a template. An amplified fragment was inserted to the retrovirus vector pMX-IRES-GFP in the same manner as described in (1) of Example 2.

SCR-3FxhoI

ccgCTCGAGccaccATGAAGCCTTTTCATACTGCC (SEQ ID NO: 32)

SCR-3Reco

tccGAATTCttattgtttgtaggtccgtgg (SEQ ID NO: 33)

(2) Preparation of stromal cells highly expressing SCR-3

AGM-s3-A7 cells in which SCR-3 was highly expressed were prepared by using the above retrovirus vector in the same manner as (2) of Example 2.

(3) Determination of activity to support hematopoietic stem cells of stromal cells in which SCR-3 is highly expressed

In the same manner as described in (III) (2) of Example 1, determination of the activity to support hematopoietic stem cells was performed except that AGM-S3-A7 cells, AGM-S3-A7 cells in which SCR-3 was highly expressed through retrovirus, and AGM-S3-A7 cells into which a control vector was introduced were seeded in a 24-well culture dish (Falcon) at 1×10^5 cells/well.

The results are shown in Fig. 6. Hematopoietic cells co-cultured with AGM-s3-A7 cells in which SCR-3 was highly expressed (A7/SCR-3) showed high chimerism in recipient individuals after the transplantation compared

with the parent cell lines or hematopoietic cells co-cultured with the cells into which a control vector was introduced. The high chimerism was observed in myeloid and lymphoid cells two months after the transplantation. Therefore, it is revealed that hematopoietic stem cells and hematopoietic progenitor cells which can reconstitute the hematopoietic system in bodies of irradiated mice have maintained and amplified superiorly to the co-culture with cells into which SCR-3 is not introduced, during the co-culture period. From the results, it is revealed that an activity of stromal cells to support survival or proliferation of hematopoietic stem cells or hematopoietic progenitor cells is increased by high expression of SCR-3. Therefore, it is revealed that a gene product of SCR-3 has an activity to affect hematopoietic stem cells or hematopoietic progenitor cells to support survival or proliferation thereof or an activity to affect stromal cells to enhance a hematopoietic cell-supporting activity of the stromal cells or impart the activity to the stromal cells.

Example 4 Cloning of SCR-4 and activity determination

By searching GenBank database for the nucleotide sequence shown in SEQ ID NO: 3 with BLAST, it has been found that SCR-4 has a high homology to *Homo sapiens* clone 25077 mRNA of an accession number AF131820, and

that SCR-4 is a mouse ortholog. This sequence is described in WO 00/66784.

The nuclotide sequence of ORF of AF131820 and the amino acid sequence deduced from the nucleotide sequence are shown in SEQ ID NO: 16. Only the amino acid sequence is shown in SEQ ID NO: 17.

The nuclotide sequence of ORF of SCR-4 and the amino acid sequence deduced from the nucleotide sequence are shown in SEQ ID NO: 14. Only the amino acid sequence is shown in SEQ ID NO: 15.

Determination of the activity of SCR-4 to support the hematopoietic stem cells or hematopoietic progenitor cells was performed as follows.

(1) Construction of retrovirus vector for expression of human SCR-4

From 3 μg of mRNA derived from fetal liver (CLONETEC, U.S.A.), cDNA was synthesized by using oligo-dT primer and reverse transcriptase (SuperscriptII, GIBCO-BRL). Using the cDNA as a template, the ORF region of human SCR-4 was amplified by PCR with HSCR-4FxhoI and HSCR-4RecoRV primers having the following nucleotide sequences. An amplified fragment was digested with *Xho*I and inserted to the retrovirus vector pMX-IRES-GFP in the same manner as described in (1) of Example 2. For the insertion, the pMX-IRES-GFP was digested with a restriction enzyme *Eco*RI, blunt-ended with KOD DNA synthase (TOYOBO, Japan) and digested with a restriction

enzyme *Xho*I.

HSCR-4FxhoI

CCGCTCGAGCCACCatgttggctgcaaggctggtgt (SEQ ID NO: 34)

HSCR-4RecoRV

CCGGATATCtcatttctttctgttgcctcca (SEQ ID NO: 35)

(2) Preparation of stromal cells highly expressing human SCR-4

AGM-s3-A9 cells in which human SCR-4 was highly expressed were prepared by using the above retrovirus vector in the same manner as (2) of Example 2.

(3) Co-culture of human hematopoietic stem cells and stromal cells highly expressing human SCR-4, and determination of proliferation statuses of hematopoietic stem cells and hematopoietic progenitor cells by clonogenic assay

In the same manner as described in (III) (1) 3) to 4) of Example 1, AGM-s3-A9 cells in which SCR-4 was highly expressed through retrovirus, AGM-s3-A9 cells into which a control vector was introduced, or AGM-s3-A9 cells were co-cultured with CD34-positive hematopoietic stem cells derived from human cord blood, and proliferation statuses of hematopoietic stem cells and hematopoietic progenitor cells are determined.

Fig. 6 shows results when the CD34-positive hematopoietic stem cells were co-cultured with AGM-S3-A9 cells in which human SCR-4 was highly expressed, AGM-S3-

A9 cells into which a control vector was introduced or AGM-S3-A9 cells for two weeks. As a result, the co-culture with AGM-S3-A9 cells in which human SCR-4 was highly expressed, increases of BFU-E and CFU-C were observed. Therefore, it has been revealed that the activity to support hematopoietic stem cells or hematopoietic progenitor cells, of AGM-S3-A9 increases by allowing human SCR-4 to be highly expressed. From the results, it has been revealed that human SCR-4 has an activity to support survival or proliferation of hematopoietic stem cells or hematopoietic progenitor cells or an activity to affect stromal cells to impart a hematopoietic cell-supporting activity to the stromal cells.

Example 5 Cloning of SCR-5 and activity determination

In the nucleotide sequence of SEQ ID NO: 4 obtained by the SBH analysis, the presence of ORF was predicted. The nuclotide sequence of ORF and the amino acid sequence deduced from the nucleotide sequence are shown in SEQ ID NO: 18. Only the amino acid sequence is shown in SEQ ID NO: 19.

By searching GenBank database for the nucleotide sequence of SEQ ID NO: 18 with BLAST, it has been found that SCR-5 has a high homology with *Homo sapiens* esophageal cancer related gene 4 portein (ECRG4) mRNA of an accession number AF325503, and that SCR-5 is a mouse

ortholog of AF325503. The nuclotide sequence of ORF of AF325503 and the amino acid sequence deduced from the nucleotide sequence are shown in SEQ ID NO: 20. Only the amino acid sequence is shown in SEQ ID NO: 21.

Determination of the activity of SCR-5 to support the hematopoietic stem cells or hematopoietic progenitor cells was performed as follows.

(1) Construction of retrovirus vector for expression of mouse SCR-5

Based on the nucleotide sequence of SCR-5 ORF, SCR-5FxhoI and SCR-5Rblunt primers having the following nucleotide sequences were prepared for retrovirus cloning, and PCR was performed using DNA having the nucleotide sequence shown in SEQ ID NO: 23 as a template. An amplified fragment was digested with a restriction enzyme *Xho*I and inserted to the retrovirus vector pMX-IRES-GFP in the same manner as described in (1) of Example 2. For the insertion, the pMX-IRES-GFP was digested with a restriction enzyme *Eco*RI, blunt-ended with KOD DNA synthase (TOYOBO, Japan) and digested with a restriction enzyme *Xho*I.

SCR-5FxhoI

ccgCTCGAGccaccatgagcacctcgtctgcgcg (SEQ ID NO: 36)

SCR-5Rblunt

tccGTTAACttaatagtcatcatagttca (SEQ ID NO: 37)

(2) Preparation of stromal cells highly expressing SCR-5

AGM-s3-A7 cells in which SCR-5 was highly expressed were prepared by using the above retrovirus vector in the same manner as (2) of Example 2.

(3) Determination of activity to support hematopoietic stem cells of stromal cells in which SCR-5 is highly expressed

In the same manner as described in (3) of Example 3, determination of the activity to support hematopoietic stem cells was performed.

The results are shown in Fig. 8. Hematopoietic cells co-cultured with AGM-s3-A7 cells in which SCR-5 was highly expressed (A7/SCR-5) showed high chimerism in recipient individuals after the transplantation compared with the parent cell lines or hematopoietic cells co-cultured with the cells into which a control vector was introduced. The high chimerism was observed in myeloid and lymphoid cells two months after the transplantation. Therefore, it is revealed that hematopoietic stem cells and hematopoietic progenitor cells which can reconstitute the hematopoietic system in bodies of irradiated mice have maintained and amplified superiorly to the co-culture with cells into which SCR-5 is not introduced, during the co-culture period. From the results, it is revealed that an activity of stromal cells to support survival or proliferation of hematopoietic stem cells or hematopoietic progenitor

cells is increased by high expression of SCR-5. Therefore, it is revealed that a gene product of SCR-5 has an activity to affect hematopoietic stem cells or hematopoietic progenitor cells to support survival or proliferation thereof or an activity to affect stromal cells to enhance a hematopoietic cell-supporting activity of the stromal cells or impart the activity to the stromal cells.

Example 6 Cloning of SCR-6 and activity determination

Based on the nucleotide sequence of SEQ ID NO: 5, a probe was prepared and AGM-s3-A9 cDNA was screened by hybridization to obtain a gene containing ORF of mouse SCR-6.

AGM-s3-A9 cells (1.4×10^8 cells) were dissolved in 20 mL of ISOGEN (Nippon gene, Japan) and total RNAs were prepared according to the attachment. Messenger RNAs were prepared from one milligram of the total RNAs according to the protocol of the mRNA purification kit (Amersham Pharmacia, U.S.A.). By using SMART cDNA library construction kit (CLONTECH, U.S.A.), cDNA libraries devided to 15 fractions were prepared from the 2 μg of the prepared mRNAs according to the attachment. The libraries contained about 400,000 of independent clones in total. For each fraction, PCR was performed under the following conditions to identify a fraction containing SCR-6 cDNA.

Based on the sequence of a partial fragment of the mouse SCR-6 gene, the following primers were prepared, and PCR was performed with 35 cycles of 94°C, 30 seconds, 55°C, 30 seconds and 72°C, 1 minute, by using each fraction of AGM-s3-A9 cDNA libraries as a template.

SCR-6F

AGCTCATTACTGTATATTTA (SEQ ID NO: 22; 1971-1990)

(SEQ ID NO: 38)

SCR-6R

GCTATATTTCATAAGTCATC (SEQ ID NO: 22; 2330-2349)

(SEQ ID NO: 39)

The PCR product was subjected to 2% agarose gel electrophoresis and a fraction from which the PCR product having the expected size was obtained was identified. For each of two fractions among the positive fractions, 50,000 plaques were seeded on two 15-cm petri dishes and incubated 37°C for 10 hours. Then, plaques of each petri dish were replicated to a sheet of Biodyne nylon filter (Pall, U.S.A.). The replicated nylon filter was subjected to DNA fixation treatment according to the attachment, and screening with ^{32}P-labeled DNA probe was performed.

The probe was prepared as follows. PCR was performed with 35 cycles of of 94°C, 30 seconds, 55°C, 30 seconds and 72°C, 1 minute, by using SCR-6F and SCR-6R and the plasmid containing a partial fragment of the

mouse SCR-6 gene as a template. The PCR product was subjected to 2% agarose gel electrophoresis and the amplified fragment was purified by JETSORB. By using 25 ng of the obtained PCR fragment, ^{32}P-labeled DNA probe was prepared with Megaprime labeling kit (Amersham Pharmacia, U.S.A.).

Hybridization and washing were performed with ExpressHybSolution (CLONETECH, U.S.A.) according to the attachment. An X-ray film was exposed to the filter and developed with a Fuji film auto developer to analyze the result. A plaque at a position corresponding to the resultant strongly exposed portion was scraped from the petri dish, and seeded again so that about 200 of plaques should appear on 10-cm petri dish. Screening was again performed according to the above-mentioned method to isolate a single plaque. The obtained phage clone was transfected to *E. coli* strain BM25.8 according to the attachment of SMART cDNA library construction kit, and allowed to be converted to plasmid in the cells to form colony on LB agar medium containing 50 μg/ml ampicilin. A single colony of the transfected *E. coli* was inoculated to 3 ml of LB medium containing 50 μg/ml ampicilin and cultured at 30°C overnight. Plasmid was extracted with RPM kit (BIO101, U.S.A.) to obtain about 10 mg of plasmid.

Sequencing the both ends of the inserted fragment with an ABI377 DNA sequencer by using λTriplEx5'LD-

Insert Screening Amplimer (CTCGGGAAGCGCGCCATTGTGTTGGT (SEQ ID NO: 40); CLONTECH, U.S.A.) revealed that it included cDNA containing the nucleotide sequence from nucleotide 1 of SEQ ID NO: 5. The full-length nucleotide sequence was also determined with the ABI377 DNA sequencer. The nuclotide sequence and the amino acid sequence deduced from a nucleotide sequence predicted as ORF in the nucleotide sequence are shown in SEQ ID NO: 22. Only the amino acid sequence is shown in SEQ ID NO: 23.

By searching the cDNA database of KAZUSA DNA Institute for mouse SCR-6 nucleotide sequence with BLAST, it has been found homologous *Homo sapiens* clone HJ08186R. HJ08186R has a high homology to the nucleotide sequence from guanine at nucleotide position 319 to adenine at nucleotide position 917 of mouse SCR-6, but is not predicted to have an entire ORF sequence.

KF305X primer; 5'- CCG CTC GAG CCG CCC AGA TGC AGT TTC GC -3' (SEQ ID NO: 49) having Xho I site at 5'-end was prepared according to the nucleotide sequence of HJ08186R, 5'- CCG CCC AGA TGC AGT TTC GC -3' (nucleotide position: 10-29 in SEQ ID NO: 49), which is homologous to predicted initial methionine coding region of mouse SCR-6. 3'-RACE was performed with KOD-PLUS- (TOYOBO #KOD201) for the DNA polymerase and the enzyme reaction system by following protocol in the package insert. Primers used for amplification were KF305X primer for

5'-end primer and AP1 primer in Marathon Ready cDNA (CLONTECH) for 3'-end primer (0.2 μM of each final concentration). Marathon Ready cDNA Human Fetal Liver (CLONTECH#7403-1) was used as a template. PCR was performed with GeneAmp PCR System 9700 (Applied Biosystems). Amplification was performed with 94°C for 5 minutes; 5 cycles of 94°C, 10 seconds, 72°C, 4 minutes; 5 cycles of 94°C, 10 seconds, 70°C, 4 minutes; 20 cycles of 94°C, 10 seconds, 68°C, 4 minutes; 72°C for 7 minutes and thereafter 4°C. By using 1/50 volume (1 μl) of the amplified product, 2nd amplification was further performed with KF305X primer for 5'-end primer and AP2 primer for 3'-end primer (0.2 μM of each final concentration). The 2nd amplification was performed with 94°C for 5 minutes; 5 cycles of 94°C, 10 seconds, 72°C, 4 minutes; 5 cycles of 94°C, 10 seconds, 70°C, 4 minutes; 35 cycles of 94°C, 10 seconds, 68°C, 4 minutes; 72°C for 7 minutes and thereafter 4°C. As a result, an amplified band of about 2 kilo base pairs was obtained.

The 2nd amplified product was incubated with dNTPs (40 μM of final concentration) and 5 units of Takara Taq (Takara Shuzo#R001A) at 72°C for 7 minutes and subjected to agarose gel electrophoresis. A DNA fragment about 2 kilo base pairs in size was identified and purified by JETSORB Gel Extraction Kit (Genomed#110150). The purified DNA fragment was inserted to the pGEM-T Easy vector (Promega) by conventional method.

The nucleotide sequences of obtained clones were determined with the ABI377 DNA sequencer (Applied Biosystems). The nucleotide sequence and amino acid sequence deduced from a nucleotide sequence predicted as ORF are shown in SEQ ID NO: 47. Only the amino acid sequence is shown in SEQ ID NO: 48. The nucleotide sequence contains a predicted ORF of 732 base pairs in size (nucleotide position: 18-749 in SEQ ID NO: 47) and has homology with the mouse SCR-6 coding region at 92.3% (nucleotide sequence) and 95.9% (amino acid sequence). Thus, the sequence was identified as a counterpart of mouse SCR-6 in human and defined as human SCR-6. The homology was determined with homology search in the compare function of DNASIS version 3.7 (Hitachi Software Engineering).

Determination of the activity of SCR-6 to support the hematopoietic stem cells or hematopoietic progenitor cells was performed as follows.

(1) Construction of retrovirus vector for expression of mouse SCR-6

Based on the nucleotide sequence of SCR-6 ORF, SCR-6FxhoI and SCR-6Reco primers having the following sequences were prepared for retrovirus cloning, and PCR was performed by using DNA having the nucleotide sequence shown in SEQ ID NO: 22 as a template. An amplified fragment was inserted to the retrovirus vector pMX-IRES-GFP in the same manner as described in (1) of

Example 2.

SCR-6FxhoI

ccgctcgagccaccATGCGTTTTTGCCTCTTCTC (SEQ ID NO: 41)

SCR-6Reco

cggaattcTTATTGGTTCACTCTGTCTG (SEQ ID NO: 42)

(2) Preparation of stromal cells highly expressing SCR-6

AGM-s3-A9 cells in which SCR-6 was highly expressed were prepared by using the above retrovirus vector in the same manner as (2) of Example 2.

(3) Co-culture of human hematopoietic stem cells and stromal cells highly expressing SCR-6, and determination of proliferation statuses of hematopoietic stem cells and hematopoietic progenitor cells by clonogenic assay

In the same manner as described in (III) (1) 3) to 4) of Example 1, AGM-s3-A9 cells in which SCR-6 was highly expressed through retrovirus, AGM-s3-A9 cells into which a control vector was introduced, or AGM-s3-A9 cells were co-cultured with CD34-positive hematopoietic stem cells derived from human cord blood, and proliferation statuses of hematopoietic stem cells and hematopoietic progenitor cells are determined.

Fig. 9 shows results when the CD34-positive hematopoietic stem cells were co-cultured with AGM-S3-A9 cells in which SCR-6 was highly expressed (A9/SCR-9), AGM-S3-A9 cells into which a control vector was introduced (A9/pMXIG) or AGM-S3-A9 cells (A9) for two

weeks. As a result, the co-culture with AGM-S3-A9 cells in which SCR-6 was highly expressed, increases of BFU-E and CFU-C were observed. Therefore, it has been revealed that the activity to support hematopoietic stem cells or hematopoietic progenitor cells, of AGM-S3-A9 increases by allowing SCR-6 to be highly expressed. From the results, it has been revealed that the gene product of SCR-6 has an activity to support survival or proliferation of hematopoietic stem cells or hematopoietic progenitor cells or an activity to affect stromal cells to enhance a hematopoietic cell-supporting activity of the stromal cells or impart the activity to the stromal cells.

Example 7 Cloning of SCR-7 and activity determination

In the nucleotide sequence of SEQ ID NO: 6 obtained by the SBH analysis, the presence of ORF was predicted. The nuclotide sequence of ORF and the amino acid sequence deduced from the nucleotide sequence are shown in SEQ ID NO: 24. Only the amino acid sequence is shown in SEQ ID NO: 25.

Determination of the activity of SCR-7 to support the hematopoietic stem cells or hematopoietic progenitor cells was performed as follows.

(1) Construction of retrovirus vector for expression of mouse SCR-7

Based on the nucleotide sequence of SCR-7 ORF, SCR-

7FsalI and SCR-7Reco primers having the following nucleotide sequences were prepared for retrovirus cloning, and PCR was performed using DNA having the nucleotide sequence shown in SEQ ID NO: 24 as a template. An amplified fragment was inserted to the retrovirus vector pMX-IRES-GFP in the same manner as described in (1) of Example 2.

SCR-7FSalI

acgcgtcgacccaccATGCCCCGCTACGAGTTG (SEQ ID NO: 43)

SCR-7Reco

attGAATTCTCACTTCTTCCTCCTCTTTG (SEQ ID NO: 44)

(2) Preparation of stromal cells highly expressing SCR-7

AGM-s3-A9 cells in which SCR-7 was highly expressed were prepared by using the above retrovirus vector in the same manner as (2) of Example 2.

(3) Co-culture of human hematopoietic stem cells and stromal cells highly expressing SCR-7, and determination of proliferation statuses of hematopoietic stem cells and hematopoietic progenitor cells by clonogenic assay

In the same manner as described in (III) (1) 3) to 4) of Example 1, AGM-s3-A9 cells in which SCR-7 was highly expressed through retrovirus, AGM-s3-A9 cells into which a control vector was introduced, or AGM-s3-A9 cells were co-cultured with CD34-positive hematopoietic stem cells derived from human cord blood, and proliferation statuses of hematopoietic stem cells and

hematopoietic progenitor cells are determined.

Fig. 10 shows results when the CD34-positive hematopoietic stem cells were co-cultured with AGM-S3-A9 cells in which SCR-7 was highly expressed (A9/SCR-7), AGM-S3-A9 cells into which a control vector was introduced (A9/pMXIG) or AGM-S3-A9 cells (A9) for two weeks. As a result, the co-culture with AGM-S3-A9 cells in which SCR-7 was highly expressed, increases of BFU-E and CFU-C were observed. Therefore, it has been revealed that the activity to support hematopoietic stem cells or hematopoietic progenitor cells, of AGM-S3-A9 increases by allowing SCR-7 to be highly expressed. From the results, it has been revealed that the gene product of SCR-7 has an activity to support survival or proliferation of hematopoietic stem cells or hematopoietic progenitor cells or an activity to affect stromal cells to enhance a hematopoietic cell-supporting activity of the stromal cells or impart the activity to the stromal cells.

Example 8 Cloning of SCR-8 and activity determination

By searching GenBank database for the nucleotide sequence shown in SEQ ID NO: 7 with BLAST, it has been found that SCR-8 is the same gene as *Mus musculus* mRNA for ADAM23 of an accession number AB009673. The nuclotide sequence of SCR-8 ORF and the amino acid sequence deduced from the nucleotide sequence are shown

in SEQ ID NO: 26. Only the amino acid sequence is shown in SEQ ID NO: 27.

Also, the sequence encoding Human MDC3 protein [*Homo sapiens*] described by JP 11155574-A has a homology of not less than 90% with SCR-8 and, therefore, is a human ortholog of SCR-8. The nuclotide sequence of this ORF and the amino acid sequence deduced from the nucleotide sequence are shown in SEQ ID NO: 28. Only the amino acid sequence is shown in SEQ ID NO: 29.

Determination of the activity of SCR-8 to support the hematopoietic stem cells or hematopoietic progenitor cells was performed as follows.

(1) Construction of retrovirus vector for expression of mouse SCR-8

Based on the nucleotide sequence of SCR-8 ORF, SCR-8FxhoI and SCR-8Reco primers having the following nucleotide sequences were prepared, and PCR was performed using AGM-s3-A9 cDNA as a template. An amplified fragment was inserted to the retrovirus vector pMX-IRES-GFP in the same manner as described in (1) of Example 2.

SCR-8FxhoI

ccgctcgagccaccATGAAGCCGCCCGGCAGCATC (SEQ ID NO: 45)

SCR-8Reco

cggaattcTCAGATGGGGCCTTGCTGAGT (SEQ ID NO: 46)

(2) Preparation of stromal cells highly expressing SCR-8

AGM-s3-A9 cells in which SCR-8 was highly expressed were prepared by using the above retrovirus vector in the same manner as (2) of Example 2.

(3) Co-culture of human hematopoietic stem cells and stromal cells highly expressing SCR-8, and determination of proliferation statuses of hematopoietic stem cells and hematopoietic progenitor cells by clonogenic assay

In the same manner as described in (III) (1) 3) to 4) of Example 1, AGM-s3-A9 cells in which SCR-8 was highly expressed through retrovirus, AGM-s3-A9 cells into which a control vector was introduced, or AGM-s3-A9 cells were co-cultured with CD34-positive hematopoietic stem cells derived from human cord blood, and proliferation statuses of hematopoietic stem cells and hematopoietic progenitor cells are determined.

Fig. 11 shows results when the CD34-positive hematopoietic stem cells were co-cultured with AGM-S3-A9 cells in which SCR-8 was highly expressed, AGM-S3-A9 cells into which a control vector was introduced or AGM-S3-A9 cells for two weeks. As a result, the co-culture with AGM-S3-A9 cells in which SCR-8 was highly expressed, increases of BFU-E and CFU-C were observed. Therefore, it has been revealed that the activity to support hematopoietic stem cells or hematopoietic progenitor cells, of AGM-S3-A9 increases by allowing SCR-8 to be highly expressed. From the results, it has been revealed that the gene product of SCR-8 has an

activity to support survival or proliferation of hematopoietic stem cells or hematopoietic progenitor cells or an activity to affect stromal cells to enhance a hematopoietic cell-supporting activity of the stromal cells or impart the activity to the stromal cells.

Industrial Applicability

A factor supporting the proliferation or survival of hematopoietic stem cells or hematopoietic progenitor cells, which is derived from the stromal cells, is provided.